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Herpes simplex virus proteins are damaged following photodynamic inactivation with phthalocyanines

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Abstract

The photodynamic inactivation of herpes simplex virus type 1 (HSV-1) by two phthalocyanines (Pcs), the cationic dye HOSiPcOSi$(CH_3)_2(CH_2)_3N^+(CH_3)_3I^-$ (Pc5) and the amphiphilic dye aluminum dibenzodisulfophthalocyanine hydroxide (AlN_2SB_2POH), has been compared with that by the anionic dye, Merocyanine 540 (Mc540). Both Pc derivatives demonstrate a remarkable virucidal activity upon light activation even 3 h after the onset of HSV-1 adsorption, while Mc540 is effective for only 30 min after adsorption. Since fusion and virus penetration are promoted by membrane glycoproteins, we have studied the damage to viral proteins following photodynamic treatment (PDT) of HSV-1 and its relation to inactivation. The effect of AlN_2SB_2POH PDT is assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Major changes are found in the protein profile of PDT-treated HSV-1. A reduced ability of specific antibodies to react with HSV-1 major envelope proteins is detected by employing the Western blot assay. In particular, we demonstrate the related changes of glycoprotein D (gD), a structural protein of the HSV envelope. Since the envelope proteins participate in viral entry into the host cell, these alterations to viral envelope proteins may impair their ability to participate in early events of viral entry, leading to reduced infectivity of HSV-1. In contrast, no significant changes in the proteins' electrophoretic mobility could be seen after PDT with Mc540 or with Pc5. When HSV-1 purified proteins are subjected to combined electrophoretic and electro-osmotic forces on cellulose acetate, there is a shift in their cathode mobility, which may indicate changes in the protein mass and protein net charges following AlN_2SB_2POH photosensitization. There are only minor changes in the virus proteins, assayed as above, when HSV-1 is treated with Pc5.

Keywords: Herpes simplex virus; Photodynamic treatment; Virus inactivation; Phthalocyanines

1. Introduction

Phthalocyanines (Pcs) are potent photosensitizers for photodynamic inactivation of viruses [1–3] and are being studied for their potential use in blood sterilization [4]. The temporal kinetics of photoinactivation during the stages of viral adsorption and penetration into host cells have been studied with the herpes simplex virus (HSV) [5–7]. We have previously shown that sensitivity to photoinactivation decreased progressively with time after the addition of herpes simplex type 1 (HSV-1) and herpes simplex type 2 (HSV-2) to their host cells [7]. The intracellular viruses were more resistant than the free virions. The loss of photosensitivity with time after infection varies for various Pc dyes. Thus, amphiphilic Pcs, which can better penetrate the plasma membrane [8], are more effective for inactivation of intracellular HSV [7]. The efficacy of inactivation of enveloped viruses by photodynamic treatment (PDT) with Pc is dependent upon the metal ligand and the extent of sulfonation [1,3].

The target of photoinactivation appears to be the virus envelope [4]. Its molecular nature has not been defined and probably differs for different photosensitizers and viruses. Merocyanine 540 (Mc540) was reported to inhibit virus adsorption and penetration into host cells [9,10]. Rose bengal and hypericin photosensitization of enveloped viruses [11,12] caused protein cross-linking, which was accompanied by inactivation of enveloped, but not of nonenveloped, viruses. Virus inactivation by Pc appears to be mediated by singlet oxygen [13].

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The envelope of HSV-1 contains at least 12 different membrane proteins [14–16]. These include the glycoproteins gB, gC, gD, gE, gH, gI, gJ, gK, gL. Glycoproteins gB, gD, gH and gL were found to potentiate viral entry [17–25]. The exact stage during entry that is affected by photodamage to the viral envelope is still unclear [7,26]. In our former study of photodamage, we suggested that damage to the viral envelope prevents adsorption and binding to cell receptors, resulting in a loss of infectivity [7].

Since sensitivity to Pc dyes, such as amphiphilic phthalocyanine AlN_2SB_2POH and $AlNSB_3POH$, persists longer after infection than for anionic Pc dyes such as $AlPcS_4$ and Mc540, it has been suggested that other steps associated with the virus life cycle following virus penetration were also sensitive to such photosensitization [7].

In the present study, we have compared AlN_2SB_2POH, $HOSiPcOSi(CH_3)_2(CH_2)_3N^+(CH_3)_3I^-$ (Pc5) and Mc540 with respect to photodynamic inactivation of HSV-1. We have investigated the involvement of viral proteins in photodynamic inactivation using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting and cellulose acetate electrophoresis.

2. Materials and methods

2.1. Photosensitizers

Aluminum dibenzodisulfophthalocyanine hydroxide (AlN_2SB_2POH) was obtained from Professor J.E. Van Lier (Sherbrooke, Canada) and dissolved in methanol: dimethylsulfoxide (DMSO) (1:2). The silicon phthalocyanine Pc5 was kindly provided by Professor M.E. Kenney (Cleveland, OH) and dissolved in DMSO. Mc540 was purchased from Sigma (Rehovot, Israel) and dissolved in 50% ethanol. All dyes were stored at 4°C as 1 mM stock solutions and sterilized by filtration.

2.2. Viruses

HSV-1, MacIntyre strain, was used in all studies reported here. The procedures for propagation and titration have been previously reported [7].

2.3. Light exposure

The light source was a slide projector equipped with a 150 W quartz halogen light bulb. For exposure of Pc-containing samples, the light was filtered by a cut-off filter ($\lambda > 605$ nm). The incident fluence rate was 200 W m^{-2}. Exposure of viral particles and cells was done in 3 ml of phosphate buffered saline (PBS), containing 2% fetal calf serum (FCS) in 35 mm polystyrene plates at 23°C for 15 min, resulting in a dose of 18 J cm^{-2}, unless otherwise specified.

2.4. Viral photoinactivation

The procedure used was described previously [7]. Briefly, Vero cells were infected with 200 μl of cell-free viral suspension at a concentration of 2.9×10^5–2×10^6, resulting in a multiplicity of infection (MOI) of one to two. The viruses were allowed to adsorb for various time intervals at 33 ± 2°C. Following adsorption, the excess virus was removed and the cells were rinsed with PBS. Dye was added and the plates were immediately exposed to light or kept in the dark for 15 min. In each experiment, infected cells without dye were included at the same experimental conditions and served as controls. Following light exposure, the plates were rinsed and overlaid with 2% methylcellulose in Eagle's minimal essential medium supplemented with non-essential amino acids (MEM-NAA) supplemented with 2% FCS. The plates were incubated at 37°C for five days in a 5% CO_2 humidified incubator. Plaques were scored and the titers calculated based on the mean count of three replicate wells.

2.5. Purification of virus particles

The procedure was performed according to Spear and Roizman [27]. Vero cells, grown in MEM-NAA containing 2% FCS, were infected with HSV-1 at an MOI of one to two for 24–30 h. The infected cells (70–80%) were scraped off the glass surface after infection and collected by low-speed centrifugation (all steps were carried out at 0–4°C). The infected cells were washed with cold PBS containing 2% FCS, then resuspended in PBS containing 2% FCS, with or without 5 μM of AlN_2SB_2POH, 5 μM of Pc5 or 25 μM of Mc540, and then immediately exposed to light or kept in the dark for 15 min. The cells were then rinsed twice with PBS and disrupted with five strokes of a Dounce homogenizer. Sucrose (60% wt./vol.) was added to yield a final concentration of 0.25 M, and the cytoplasm was separated from the nuclei by centrifugation at 1500 rpm for 10 min in a PR-2 International refrigerated centrifuge. A sample of 2 ml cytoplasm was layered on a 17 ml Dextran T10 gradient (10–30%) made up in 1 mM phosphate buffer and 1 mM phenylmethylsulfonylfluoride (PMSF). The gradients were centrifuged for 90 min at 20 000 rpm in a Beckman SW 27 rotor. The virions that were found in a diffused light-scattering band contained purified enveloped viruses (confirmed by electron microscopy). The Dextran band was diluted fourfold with 0.01 M Tris buffer saline (TBS) pH 7.4 and then pelleted by centrifugation at 25 000 rpm for 2 h in a SW 27 rotor. The pellets were suspended in a small volume of 0.01 M TBS containing 1 mM PMSF and stored at −70°C for analysis by SDS-PAGE.

2.6. SDS-PAGE

SDS-PAGE was performed according to the standard procedure of Laemmli [28]. A 7.5% wt./vol. solution of acrylamide cross-linked with bisacrylamide and a stacking

gel of 3.5% wt./vol. were employed and 60 μg of HSV-1 proteins (Bradford assay, Bio Rad, Hercules, CA) that were treated with PDT or were kept in the dark were loaded on each gel. Electrophoresis was performed at a constant voltage of 60 V on gels of 0.75 mm thickness and 14 cm length for 16 h. The gels were then equilibrated for 30 min in transfer buffer and then transferred to nitrocellulose at 200 mA for 2 h [29]. Gels were stained with Coomassie brilliant blue for 20 min and then destained in several changes of 7% acetic acid and 30% methanol over a period of two days. Western blot assay molecular weight (MW) markers (200 to 14 kDa, GIBCO BRL, Life Technologies, Inc. USA) were run concurrently to allow assignment of MW to the major bands.

2.7. Western blot assay

Blots were blocked with 3% BSA and probed either with mouse and HSV-1 envelope protein (33-503-09 abV, Immuneresponse, Inc., Datty, NH) or with anti-gD (33-503-10a, abV, Immuneresponse Inc., Datty, NH) diluted at 1:80 in PBS containing 1% BSA and 0.05% Tween 20 (PBS–Tween) overnight at 4°C. The blots were rinsed three times (15 min each) in PBS–Tween and incubated for 1 h with rabbit anti-mouse (P260 Dako, Denmark) peroxidase conjugated. The blots were then rinsed several times in PBS. The nitrocellulose was rinsed several times in PBS–Tween and then in TBS (150 mM NaCl and 50 mM Tris–Cl, pH 7.5) and stained with ECL reagent (Western blot chemiluminescence reagents, Sigma, Rehovot, Israel) for 1 min; membranes were then placed in a plastic sheet protector and exposed to autoradiography.

2.8. Electrophoresis on cellulose acetate

Purified virion proteins (2–3 ml) were applied to Titan III cellulose acetate plates (Helena Laboratories, Beaumont, TX) as described by Tiselius [30]. After electrophoresis for 15 min at 180 V, the plates were placed in 40–50 ml of Ponceau S stain for 6 min, then destained in 5% acetic acid and dehydrated in absolute methanol. The excess solution was discarded and the plate, with acetate side up, was placed on a blotter and into a laboratory drying oven with forced air at 50–60°C for 15 min. The plates were scanned in a densitometer, using a 525 nm filter.

3. Results

3.1. Viral photoinactivation

The photodynamic activity of AlN_2SB_2POH, Pc5 and Mc540 against HSV-1 was studied. The cytotoxicity of each compound was determined to be less than 10 in the dark. Photodynamic inactivation of HSV-1 at various times after adsorption is shown in Fig. 1. Complete inactivation ($\geq$5log10) was demonstrated at time zero and up to 30 min after adsorption following treatment with Mc540. Photodynamic inactivation was reduced at 60 min and completely disappeared at 3 h. AlN_2SB_2POH and Pc5 showed full photodynamic inactivation ($\geq$5log10) even 3 h after adsorption. Neither light alone nor dyes in the dark caused virus inactivation. These results support and extend our previous work [7].

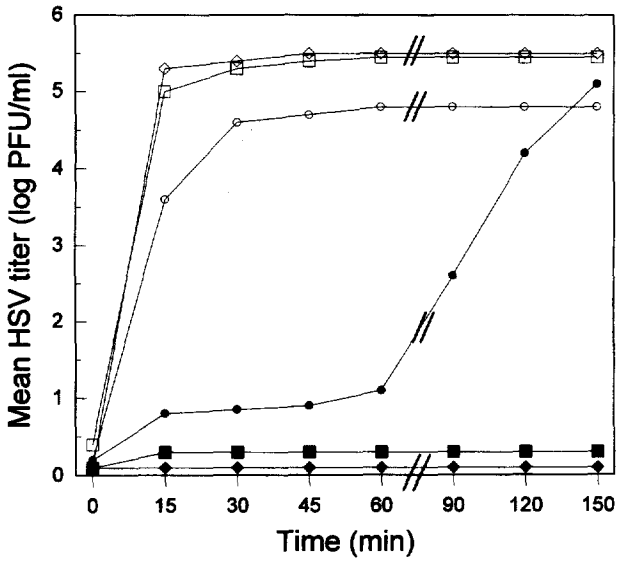

Fig. 1. Photosensitized inactivation of HSV-1 as a function of time after viral adsorption to Vero cells. At the time indicated, 5 μM of AlN_2SB_2POH (♦), 5 μM Pc5 (■) or 25 μM Mc540 (●) were added and the plates were exposed to light for 15 min. Control HSV-1-infected Vero cells were treated with the same dyes and were kept in the dark (open symbols). For details see Section 2.4.

3.2. Photodynamic damage to HSV-1 proteins

In order to study the mechanism of PDT damage to HSV-1, we examined the involvement of viral proteins in the photodynamic inactivation. Fig. 2 illustrates only minor qualitative changes in the protein profile of HSV-1 following PDT with Mc540, although the treatment could have caused some degradation of the virions leading to a general quantitative change. Likewise, there were only minor changes in the electrophoretic mobility of HSV-1 proteins after PDT with Pc5 (Fig. 3(b)). On the other hand, major differences in the profile of the viral proteins were seen after PDT with AlN_2SB_2POH (Fig. 3(a)). These reflected loss of many proteins and the appearance of cross-linked material not entering the gel (not shown).

3.3. Identification of HSV-1 envelope proteins damaged after PDT

Because major changes were seen in HSV-1 proteins after AlN_2SB_2POH PDT, further studies of the proteins were done using the Western blot assay. Specific antibodies directed to the major envelope proteins demonstrated loss of reactivity with some HSV-1 envelope proteins after AlN_2SB_2POH PDT (Fig. 4(a), lane 2). This effect was quantified using a scanning densitometer to measure the relative amount of proteins in each band (Fig. 4(b)).

Glycoprotein D (gD) is a structural component of the HSV-1 envelope which is essential for the virus penetration

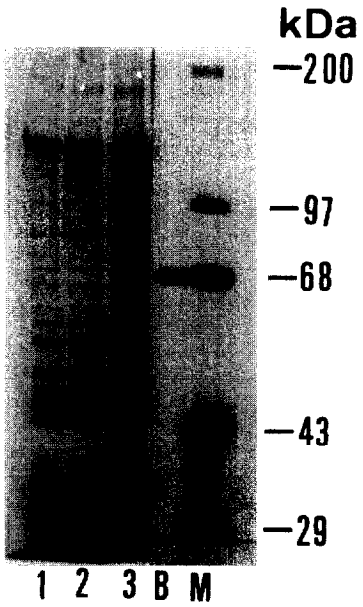

Fig. 2. HSV-1 protein profile analysed with SDS-PAGE. HSV-1 virions from infected Vero cells were purified on Dextran T10 (10–30%) gradient made in 1 mM phosphate buffer, pH 7.4, as described in Section 2.5. After analysis by SDS-PAGE, the pattern of the viral proteins was visualized by Coomassie brillant blue. Lane M, MW markers 200 to 14 kDa; lane B, bovine serum albumin; lane 1, HSV-1 was treated with 25 μM Mc540 in the dark; lane 2, as in lane 1 plus 15 min light; lane 3, untreated HSV-1.

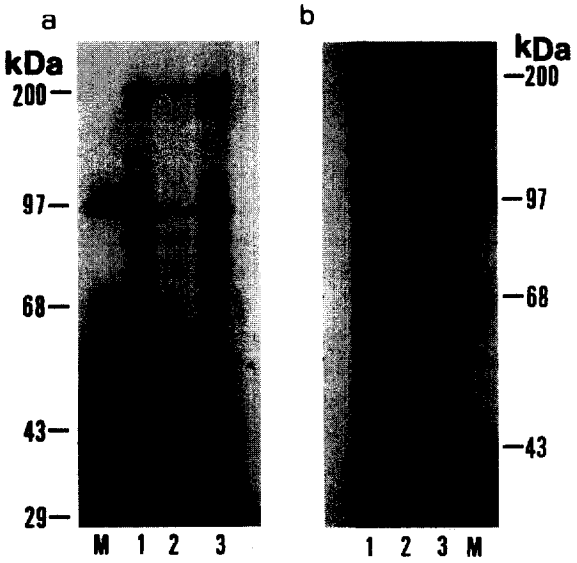

Fig. 3. (a) HSV-1 protein profile after PDT with AlN_2SB_2POH. Lane M, MW markers 200 to 14 kDa; lane 1, untreated HSV-1 control; lane 2, 5 μM AlN_2SB_2POH and light for 15 min; lane 3, 5 μM AlN_2SB_2POH in the dark. (b) HSV-1 protein profile after PDT. Lane M, MW markers 200 to 14 kDa; lane 1, HSV-1 control; lane 2, 5 μM Pc5 PDT and light for 15 min; lane 3, 5 μM Pc5 in the dark.

process. The function of this protein is highly dependent on its structure. Using specific monoclonal antibodies, we could demonstrate unequivocally that PDT with AlN_2SB_2POH caused structural changes due to dimerization or cross-linking to a protein with a MW of about 56 kD, corresponding to HSV-1 gD (Fig. 5, lane 1), as compared to the untreated control of HSV-1 gD (Fig. 5, lane 2).

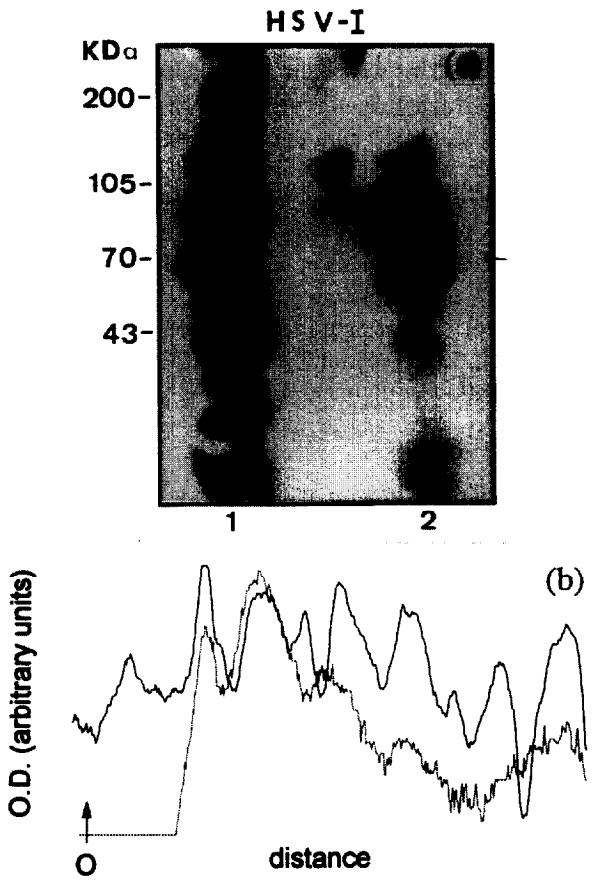

Fig. 4. Western blot assay of the major HSV-1 envelope proteins following PDT with AlN_2SB_2POH. (a) Lane 1, untreated HSV-1 envelope proteins after reaction with specific antibodies; lane 2, PDT with AlN_2SB_2POH. (b) The density of each protein band was quantified by scanner/computer analysis. For details see Section 2.7.

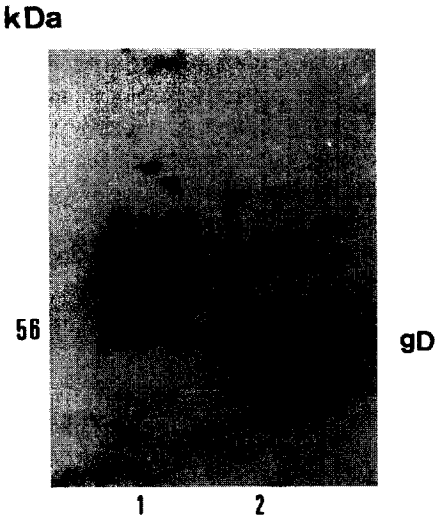

Fig. 5. Western blot assay using specific antibodies to gD. Lane 1, HSV-1 treated with AlN_2SB_2POH PDT; lane 2, untreated HSV-1.

3.4. Protein pattern on cellulose acetate electrophoresis

HSV-1 virions purified on Dextran T10 were analyzed for overall electrical charge by the Titan III cellulose acetate gel

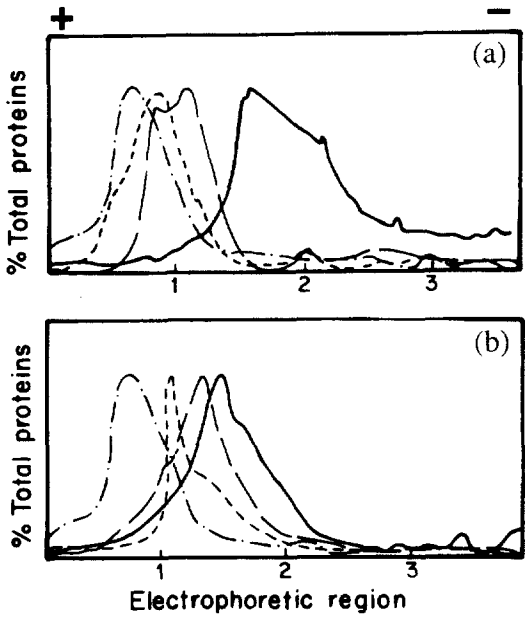

Fig. 6. Electrophoresis on cellulose acetate of purified HSV-1 proteins. (a) Control, untreated (dot–dashed curve), exposure to light only (dashed curve); $Al_2N_2SB_2POH$ in the dark (dotted curve); $Al_2N_2SB_2POH$ and light (solid curve). (b) As (a) with Pc5 as a photosensitizer.

electrophoresis, which separates HSV-1 proteins according to their respective electrical charges at pH 8.8 (Fig. 6). Both electrophoretic and electroendo-osmotic forces contribute to protein mobility under these conditions. Our results demonstrate a major shift of the proteins towards the cathode after PDT with AlN_2SB_2POH (Fig. 6(a)). Minor but significant changes were observed when HSV-1 was phototreated with Pc5 (Fig. 6(b)).

4. Discussion

Using HSV-1 as a model of lipid-enveloped viruses, we have characterized some of the targets of photodynamic inactivation. The mechanism of photoinactivation of viruses is not yet completely understood. Previous studies have shown that Mc540 binds to the viral envelope and that photoexcitation leads to structural damage of the viral envelope [10]. This is supported by lines of evidence, e.g., dye partition into membrane, fluorescence quenching of bound dye, energy transfer experiments, viral adhesion to host cells and electron microscopy of photoinactivated viruses [10]. Our earlier experiments using the amphiphilic photosensitizers $AlNSB_3POH$ and AlN_2SB_2POH demonstrated an effective inactivation of HSV-1 and HSV-2 with little or no damage to the host cells, while adenovirus, a nonenveloped virus, was not inactivated. The HSV envelope was found to be the major target for photodynamic damage following Pc inactivation [7]; hence, it is assumed that impressive viral inactivation follows dye penetration into the cell and partition into membranes, and that the effect is mainly on membrane proteins.

Protein analysis by SDS-PAGE and Western blot assay, after phototreatment with $AlNSB_3POH$ (data not shown) and AlN_2SB_2POH (Figs. 3 and 4) revealed irreversible changes in the viral envelope proteins. These alterations are in all likelihood contributing to inactivation of HSV-1. On the other hand, after phototreatment of HSV-1 with Pc5 and Me540, such major alterations in the HSV-1 protein profile were not observed.

Another observation was that alterations occur in the molecular mass and molecular charges of HSV-1 proteins. This could be seen from the cellulose acetate electrophoresis following AlN_2SB_2POH PDT (Fig. 6(a)). Further analysis of purified viral proteins by SDS-PAGE demonstrated unequivocally that these proteins had been altered after this treatment. Western blot analysis, using specific antibodies directed against HSV-1 envelope proteins, provided the evidence that PDT results in changes of epitopes in envelope proteins, and prevents their reactivity towards antibodies of approximately 56 kD. By employing monoclonal antibodies specific for gD, we were able to demonstrate that gD, which is essential for HSV-1 entry into the host cell, was damaged. Since bands heavier than the original band appeared after electrophoresis, we suggest dimerization or cross-linking as a possible mechanism. Changes in protein mass and protein net charges were seen after PDT. Unlike the case of AlN_2SB_2POH PDT, photosensitization with MC540 and Pc5 revealed only minor changes in the HSV-1 protein profile and minor changes in protein mass and net charges. Protein cross-linking and viral envelope damage were demonstrated for rose bengal and hypericin after PDT of vesicular stomatitis virus (VSV), influenza and Sendai viruses [11,12].

Abe and Wagner [31] observed that methylene blue (MB) and $AlPcS_4$ photoinactivated bacteriophage M13 and VSV in a dose-dependent manner and caused strand breaks in M13 DNA, with little alteration of viral proteins on SDS-PAGE (at doses inactivating 4.5log10 by MB or 3.5log10 by $AlPcS_4$). These authors suggested that nucleic acids may be an important target for PDT inactivation in their model viruses. However, a more recent study has shown that as Pc4 PDT but not AlPcS4 PDT caused cross-linking of a protein in the VSV envelope [32], no causative link between this damage and loss of infectivity was established. Unpublished studies from our laboratory show that the viral genome probably is not the target for $AlNSB_3POH$ and Pc5 photodynamic inactivation, which is consistent with previous studies using Pc4 PDT for HIV inactivation [33]. However $AlPcS_4$ PDT did cause oxidative damage in guanine moieties of VSV RNA [34], consistent with a previous report [31].

In conclusion, the different photosensitizers studied differ in their mechanism of virucidal action. This is supported by previous findings with other photosensitizers [31]. Our results also add to the accumulated data showing that early events of HSV-1 entry into the host cell are a complex process which requires the coordinated participation of multiple cellular and viral components. Disruption of the envelope proteins, especially gD, appeared to be sufficient to inhibit

infectivity after AlN_2SB_2POH PDT. Since the latter treatment is also effective after infection has occurred, a process during viral replication and/or assembly must also be affected. Our data, however, do not address this aspect. The inactivation mechanism of HSV-1 by Pc5 and Mc540 has not been elucidated in this work. Damage to the viral genome appears to be an unlikely candidate, but oxidative damage in the membrane lipids remains a possibility.

5. Abbreviations

AlN_2SB_2POH	aluminum dibenzodisulfophthalocyanine hydroxide
FCS	fetal calf serum
HSV-1	herpes simplex virus type 1
Mc540	Merocyanine 540
MEM-NAA	Eagle's minimal essential medium supplemented with non-essential amino acids
PBS	phosphate buffered saline
Pc	phthalocyanine
MOI	multiplicity of infection
Pc5	silicon phthalocyanine
PDT	photodynamic treatment
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

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